

AMENDMENTS TO THE SPECIFICATION

IN THE TITLE:

Please amend the title as follows:

~~SPECIFIC SUBSTRATE~~ POLYPEPTIDES FOR ~~ENZYMES CLEAVING VON~~
~~WILLEBRAND FACTOR AND METHOD FOR ASSAYING ACTIVITY THEREOF~~
CLEAVING PROTEASE ADAMTS-13

IN THE SPECIFICATION:

Please amend the specification as follows:

Please amend the paragraph at page 3, line 14 to:

ADAMTS-13 specifically cleaves the peptide bond between ~~T~~¹⁶⁰⁵yr-Tyr¹⁶⁰⁵-Met¹⁶⁰⁶ of the VWF subunit⁽²⁾. It is not known that enzymes other than ADAMTS-13 specifically cleave this site. At present, there are known methods for measuring ADAMTS-13 activity, such as (i) combinations of electrophoresis and western blot of reaction solutions using purified VWF as the substrate⁽³⁾, (ii) measurement of the ability of VWF to bind to collagen⁽⁴⁾, (iii) quantitative determination using VWF site-specific monoclonal antibodies⁽⁵⁾. However, these methods have disadvantages, for example, of requiring much time and skill for their operation, lacking the quantitateness, and having low sensitivity, and also lack the simplicity and the capability of processing many samples, making it difficult for them to become used widely in the clinical field. In addition, it is said that it is impossible in the case of ADAMTS-13, due to the problem of the substrate specificity, to utilize chromogenic or fluorescent synthetic peptide substrates which are commonly used as high throughput systems for assaying of protease activities⁽⁶⁾.

Please amend the paragraph at page 9, lines 5 to 13 to:

Brief Description of the Drawings

Fig. 1 shows the results of reactions of GST-Asp¹⁴⁵⁹-Arg¹⁶⁶⁸-H(GST-(SEQ ID NO: 2)-H), GST-Glu¹⁵⁵⁴-Arg¹⁶⁶⁸-H(GST-(SEQ ID NO: 3)-H), GST-Asp¹⁵⁸⁷-Arg¹⁶⁶⁸-H(GST-(SEQ ID NO: 4)-H), GST-Asp¹⁵⁹⁶-Arg¹⁶⁶⁸-H(GST-(SEQ ID NO: 5)-H), and GST-Asp¹⁵⁹⁶-Arg¹⁶⁵⁹-H(GST-(SEQ ID NO: 6)-H) with normal plasma at 37°C for 2 hours, followed by separation on SDS-PAGE and western blot employing an anti-GST antibody as the primary antibody.

Fig. 2 shows the substrate specificity and reaction quantitiveness of ~~GST-Asp¹⁴⁵⁹-Arg¹⁶⁶⁸-H~~ GST-Asp¹⁵⁹⁶-Arg¹⁶⁶⁸-H (GST-(SEQ ID NO: 5)-H). The reaction conditions were the same as in Fig. 1.

Please amend the paragraph at page 28, line 14 to:

RT-PCR was carried out using, as the template, RNA extracted from commercially available human umbilical ~~vein terminalothelial~~ vein endothelial cells, to obtain cDNA coding for the Asp¹⁴⁵⁹-Arg¹⁶⁶⁸ region of the VWF subunit (SEQ ID NO: 2). The sense-direction primer used was 5'-cgggatccGACCTTGCCCCTGAAGCCCCTC-3' (SEQ ID NO: 7) and the antisense-direction primer was 5'-ggaattcTCAGTGATGGTGGTGGTGCCTCTGCAGCACCAGGTCAGGA-3' (SEQ ID NO: 8) (the portions of lower case letters represent restriction enzyme recognition sites added for subcloning). The antisense-direction primer has a 6xHis tag sequence added thereto. The PCR product was digested with BamHI and EcoRI and then inserted into the *E. coli* expression vector

Please amend the paragraph at page 29, line 12 to:

Smaller polypeptides than the above-described polypeptide are more suitable for production by recombinant methods using *E. coli* or others. In order to obtain cDNAs coding the Glu¹⁵⁵⁴-Arg¹⁶⁶⁸ (SEQ ID NO: 3), Asp¹⁵⁸⁷-Arg¹⁶⁶⁸ (SEQ ID NO: 4), Asp¹⁵⁹⁶-Arg¹⁶⁶⁸ (SEQ ID NO: 5), and Asp¹⁵⁹⁶-Arg¹⁶⁵⁹ (SEQ ID NO: 6) regions, three sense-direction primers 5'-cgggatccGAGGCACAGTCCAAAGGGGACA-3' (SEQ ID NO: 9), 5'-cgggatccGACCACAGCTTCTTGGTCAGCC-3' (SEQ ID NO: 10), and 5'-cgggatccGACCGGGAGCAGGCGCCCAACC-3' (SEQ ID NO: 11), and one antisense-direction primer 5'-cggaattcTCAGTGATGGTGATGGTGATGTCGGGGGAGCGTCTCAAAGTCC-3' (SEQ ID No: 12) were employed. They were combined and processed in a similar way to produce plasmids allowing the expression of four fusion proteins, GST-Glu¹⁵⁵⁴-Arg¹⁶⁶⁸-H(GST-(SEQ ID NO: 3)-H), GST-Asp¹⁵⁸⁷-Arg¹⁶⁶⁸-H(GST-(SEQ ID NO: 4)-H), GST-Asp¹⁵⁹⁶-Arg¹⁶⁶⁸-H(GST-(SEQ ID NO: 5)-H), and GST-Asp¹⁵⁹⁶-Arg¹⁶⁵⁹-H(GST-(SEQ ID NO: 6)-H). Each of

these expression vectors was introduced into *E. coli* strain BL21, which in turn was subjected to transient expression by IPTG induction, followed by purification through nickel-affinity chromatography and glutathione-affinity chromatography to obtain each of the fusion proteins.

Please amend the paragraph at page 30, line 8 to:

When the five fusion proteins thus produced, GST-Asp¹⁴⁵⁹-Arg¹⁶⁶⁸-H(GST-(SEQ ID NO: 2)-H), GST-Glu¹⁵⁵⁴-Arg¹⁶⁶⁸-H(GST-(SEQ ID NO: 3)-H), GST-Asp¹⁵⁸⁷-Arg¹⁶⁶⁸-H(GST-(SEQ ID NO: 4)-H), GST-Asp¹⁵⁹⁶-Arg¹⁶⁶⁸-H(GST-(SEQ ID NO: 5)-H), and GST-Asp¹⁵⁹⁶-Arg¹⁶⁵⁹-H(GST-(SEQ ID NO: 6)-H), are specifically cleaved by ADAMTS-13, that is, when the site corresponding to the site between Tyr¹⁶⁰⁵-Met¹⁶⁰⁶ of the VWF subunit is cleaved, these fusion proteins will be separated into two fragments of 43.1 kDa (including the GST portion) and 7.7 kDa (including the His6 tag sequence portion), of 32.7 kDa and 7.7 kDa, of 29.0 kDa and 7.7 kDa, of 28.0 kDa and 7.7 kDa, and of 28.0 kDa and 6.7 kDa, respectively.

Please amend the paragraph at page 31, line 3 to:

In the case of the two-hour reaction, the expected fragment (indicated by the arrowheads in the figure) was clearly yielded for GST-Asp¹⁵⁸⁷-Arg¹⁶⁶⁸-H(GST-(SEQ ID NO: 4)-H) and GST-Asp¹⁵⁹⁶-Arg¹⁶⁶⁸-H(GST-(SEQ ID NO: 5)-H), while a very faint band was produced at the expected position, also for GST-Glu¹⁵⁵⁴-Arg¹⁶⁶⁸-H(GST-(SEQ ID NO: 3)-H) having a longer region. It proved that GST-Asp¹⁴⁵⁹-Arg¹⁶⁶⁸-H(GST-(SEQ ID NO: 2)-H) having a further longer region and GST-Asp¹⁵⁹⁶-Arg¹⁶⁵⁹-H(GST-(SEQ ID NO: 6)-H) having a shorter region did not give the fragment or was difficult to give the fragment. These results suggested that GST-Asp¹⁵⁸⁷-

Arg¹⁶⁶⁸-H(GST-(SEQ ID NO: 4)-H) and GST-Asp¹⁵⁹⁶-Arg¹⁶⁶⁸-H(GST-(SEQ ID NO: 5)-H) be suitable as a substrate for ADAMTS-13.

Please amend the paragraph at page 31, line 16 to:

In order to examine the specificity of GST-Asp¹⁵⁹⁶-Arg¹⁶⁶⁸-H (GST-(SEQ ID NO: 5)-H) as the substrate, among the particularly preferable substrate polypeptides for ADAMTS-13 obtained in Section B, it was reacted with plasma samples from TTP-patient family members. The reaction conditions and detection method were the same as described above. The results are shown in Fig. 2.

Please amend the paragraph at page 31, line 23 to:

When each of plasma samples from two patients was reacted with GST-Asp¹⁵⁹⁶-Arg¹⁶⁶⁸-H(GST-(SEQ ID NO: 5)-H), there was not detected the fragment which is yielded by the reaction with normal plasma (indicated by the arrowhead in the figure). On the other hand, for plasma samples from mother and elder sister of family A, and from father and mother of family B which were found to have about one-half of the activity of ADAMTS-13 in normal plasma by another method, the fragment was yielded at smaller amounts than with the normal plasma. For a plasma sample from father of family A which was found to have an even lower activity, the fragment was yielded only at a further reduced amount. These results suggest that GST-Asp¹⁵⁹⁶-Arg¹⁶⁶⁸-H(GST-(SEQ ID NO: 5)-H) is a specific artificial substrate which is cleaved quantitatively by ADAMTS-13 in plasma and is not cleaved by other enzymes.

Please amend the sentence at page 34, line 19 to:

SEQ ID NO: 7 depicts the nucleotide sequence of the sense primer used for producing Asp¹⁴⁵⁹-Arg¹⁶⁶⁸(SEQ ID NO: 2) a substrate polypeptide for ADAMTS-13 of the present invention.

Please amend the sentence at page 34, line 22 to:

SEQ ID NO: 8 depicts the nucleotide sequence of the anti-sense primer used for producing Asp¹⁴⁵⁹-Arg¹⁶⁶⁸(SEQ ID NO: 2), a substrate polypeptide for ADAMTS-13 of the present invention.

Please amend the sentence at page 34, line 25 to:

SEQ ID NO: 9 depicts the nucleotide sequence of the sense primer used for producing Glu¹⁵⁵⁴-Arg¹⁶⁶⁸(SEQ ID NO: 3), Asp¹⁵⁸⁷-Arg¹⁶⁶⁸(SEQ ID NO: 4), Asp¹⁵⁹⁶-Arg¹⁶⁶⁸(SEQ ID NO: 5), and Asp¹⁵⁹⁶-Arg¹⁶⁵⁹(SEQ ID NO: 6), substrate polypeptides for ADAMTS-13 of the present invention.

Please amend the sentence at page 35, line 4 to:

SEQ ID NO: 10 depicts the nucleotide sequence of the sense primer used for producing Glu¹⁵⁵⁴-Arg¹⁶⁶⁸(SEQ ID NO: 3), Asp¹⁵⁸⁷-Arg¹⁶⁶⁸(SEQ ID NO: 4), Asp¹⁵⁹⁶-Arg¹⁶⁶⁸(SEQ ID NO: 5), and Asp¹⁵⁹⁶-Arg¹⁶⁵⁹(SEQ ID NO: 6), substrate polypeptides for ADAMTS-13 of the present invention.

Please amend the sentence at page 35, line 8 to:

SEQ ID NO: 11 depicts the nucleotide sequence of the sense primer used for producing Glu¹⁵⁵⁴-Arg¹⁶⁶⁸(SEQ ID NO: 3), Asp¹⁵⁸⁷-Arg¹⁶⁶⁸(SEQ ID NO: 4), Asp¹⁵⁹⁶-Arg¹⁶⁶⁸(SEQ ID NO: 5), and Asp¹⁵⁹⁶-Arg¹⁶⁵⁹(SEQ ID NO: 6), substrate polypeptides for ADAMTS-13 of the present invention.

Please amend the sentence at page 35, line 12 to:

SEQ ID NO: 12 indicates the nucleotide sequence of the anti-sense primer used for producing Glu¹⁵⁵⁴-Arg¹⁶⁶⁸(SEQ ID NO: 3), Asp¹⁵⁸⁷-Arg¹⁶⁶⁸(SEQ ID NO: 4), Asp¹⁵⁹⁶-Arg¹⁶⁶⁸(SEQ ID NO: 5), and Asp¹⁵⁹⁶-Arg¹⁶⁵⁹(SEQ ID NO: 6), substrate polypeptides for ADAMTS-13 of the present invention.